
Circular dichroism studies of the B→A conformational transition in seven small DNA restriction fragments containing the Escherichia coli lactose control region

Wolfgang Hillen and Robert D. Wells

University of Wisconsin, Department of Biochemistry, College of Agricultural and Life Sciences, Madison, WI 53706, USA

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ABSTRACT

The B→A conformational transition caused by high ethanol concentrations was studied for seven DNA restriction fragments with overlapping and known sequences. Since the DNAs are homogeneous and range in GC content from 44 - 63%, they permit an evaluation of the influence of DNA sequence and base composition on the B→A transition. Moreover, their small size (80-301 bp) minimizes precipitation artifacts. The B- form spectra (in low salt) and the transition toward the C- form (in ethanol concentrations below the B→A transition) agree with prior measurements on chromosomal DNAs and are similar for all seven DNAs. At higher ethanol concentrations (80%), all fragments undergo a transition to the A- form as judged by the large increase of the positive CD band at 270 nm. Difference spectra among the fragments reveal minor differences between the A- form spectra. The ethanol concentration necessary to cause this transition is $72 \pm 2\%$ for all fragments, thus excluding a preference of the CAP-, *E. coli* RNA polymerase-, or *lac* repressor-binding sequences for the A- form. The kinetics of the B→A transition in 80% ethanol are biphasic; the initial rapid transition is an intramolecular B→A form shift and the slower transition is an aggregation (but not precipitation) of the DNA.

INTRODUCTION

The basis of gene regulation at the molecular level, namely the interaction of a regulatory protein with its relatively small DNA target site, is only poorly understood despite the availability of a substantial amount of sequence information¹⁻⁴. The possible roles of the conformations of DNAs^{3,5} as well as proteins⁴ in these processes have recently been reviewed. One problem with most prior studies has been the large amount of non-specific DNA surrounding the small amount of the specific recognition sequence; this generally

gives rise to a large background of non-specific interactions^{4,6,7}. The combined utilization of recombinant DNA techniques and high pressure liquid chromatography on RPC-5^{3,8-11} for the preparation of large amounts of homogeneous restriction fragments has provided new molecules for more sophisticated determinations.

Prior studies have indicated that the binding of specific regulatory proteins to DNA may also influence DNA conformations. For example, in the case of the interaction of the lac repressor with the lac operator, evidence suggests that the DNA undergoes a conformational change upon binding^{3,12}. Namely, the winding angle^{13,14} and the base tilting¹⁵ may be affected. The large increase in the CD signal at 270 nm of bacteriophage λ DNA, calf thymus DNA, and $(dA-dT)_n \cdot (dA-dT)_n$ upon repressor binding suggests a possible shift of the helical geometry towards the A conformation^{15,16}. Therefore, it is not only interesting to study the DNA conformation of regulatory sites, but also the conditions leading to conformational transitions in those sequences.

The majority of prior CD measurements on DNA were performed on chromosomal DNAs which give composite spectra due to the large number of sequences present. Nevertheless, several conformational transitions in DNA have been characterized by changes in the amplitude and shape of their CD spectra¹⁷. By increasing the salt concentration, the amplitude of the positive CD band decreases. This has been interpreted as a shift in the DNA helix toward the C-form^{18,19}, but this interpretation has recently been questioned²⁰. Upon dehydration by increasing the ethanol concentration, the DNA undergoes a cooperative transition from the B- to the A-form at approximately 73% ethanol²¹⁻²⁴. It has been suggested that this transition is accompanied by an aggregation of the DNA²⁵. On the other hand, studies with DNA in 80-82% ethanol oriented in flow cells revealed that no aggregation occurs under these conditions²⁶. Studies on condensed DNA samples derived from increasing the salt concentration in ethanolic DNA solutions show that the ethanol concentration needed for the B \rightarrow A transition is only a little lower than the concentration leading to condensed

forms^{27,28}. Two conditions, namely extremely low ionic strength (below 1 mM) and a low molecular weight of the DNA seem to make aggregation unlikely.

We have used a set of DNA fragments with known sequences originating from the E. coli lactose control region to study the B→A transition in homogeneous samples having low molecular weight (from 80 bp to 301 bp). The homogeneity of the DNA fragments should simplify the interpretation of physical measurements and also reveal unambiguously any sequence specific behavior. Due to the origin of these fragments in the operon, they contain as many as three specific binding sites for different proteins, namely the CAP protein, the E. coli RNA polymerase and the lac repressor (Fig. 1). Since the fragments are short, the relative concentration of the protein binding sites is high enough to reveal unique structures, if they exist. Also, any preference among the sequences for the A-conformation should be detected.

MATERIALS AND METHODS

a) DNA samples

The 95 and 301 bp fragments were prepared essentially as described⁸⁻¹⁰. The 203 bp fragment was a gift of Dr. S.C. Hardies. The 80 and 219 bp fragments were produced by cleavage of the 301 bp fragment with Hpa II and the two reaction products were separated by RPC-5 column chromatography¹⁰. The 101 and 188 bp fragments were prepared similarly using Hinf I to digest the 301 bp fragment. The restriction endonucleases were either prepared from the respective strains²⁹ (Hpa II, Eco RI, Hae III) or were purchased from BRL, Bethesda, Maryland. The DNA fragments were analyzed on polyacrylamide gels³⁰ and dialyzed in several steps to yield a final salt concentration of 2 mM sodium phosphate pH 7.3; 0.1 mM EDTA (hereafter called "standard buffer"). The DNA concentration in these stock solutions was approximately 50 µg/ml. The desired ethanol concentrations were obtained by mixing the DNA stock solution with the proper amount of 100% ethanol. All ratios of standard buffer/ethanol solutions are given in v/v.

b) Optical measurements

A Jasco Model J-41C spectropolarimeter was used to measure the ellipticity of the samples. The instrument was calibrated with a 1 mg/ml dl0- camphor-sulfonic acid solution with a path-length of 1 cm to give 0.3133 deg at 290 nm. UV spectra were measured with a Cary 15. DNA concentrations were usually below 0.6 A₂₆₀/ml and in no case exceeded 1 A₂₆₀/ml. The extinction coefficients of the DNA fragments were estimated from their GC content by linear interpolation between the values given in ref. 22 for four chromosomal DNAs with GC contents ranging from 72% to 31%. The extinctions were assumed to be constant with increasing ethanol concentration, which may lead to small errors in the amplitude of the CD signal²⁷. For the calculation of $\Delta\epsilon$, the DNA concentration was expressed as molarity phosphate. All spectra were determined at room temperature (21-23°).

c) Other methods

Sedimentation coefficients were determined using a Beckman model E analytical ultracentrifuge as described previously³¹ by following the boundary of the sedimenting DNA. The s-value for the 95 bp fragment under the conditions of ref. 31 was 4.31 which agrees well with the value predicted from its molecular weight³².

Other materials and methods and characterization of some of the fragments were previously reported⁸⁻¹¹.

RESULTS

a) Description of the fragments

Fig. 1 shows the genetic and restriction map of the 301 bp fragment and its subfragments used in this study. The GC content of the fragments studied are in brackets. The Hae III and Alu I maps are shown to define the location of the 203 and 95 bp fragments, respectively. These two fragments, as well as the 301 bp fragment, were directly isolated as described⁸ from the recombinant plasmids pRW2^{8,11}, pRW4⁸, and pRW501⁹, respectively. Thus, they contain Eco RI "sticky ends" due to their method of construction¹¹.

The subfragments of the 301 bp fragment (Fig. 1) contain

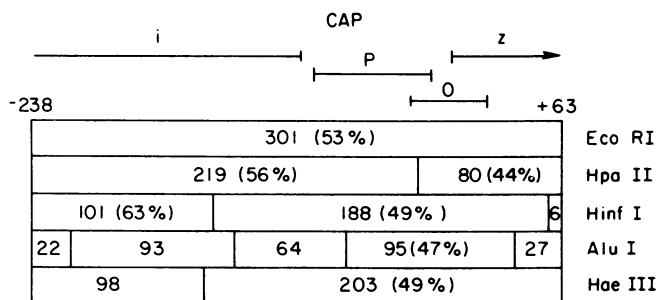


Figure 1. Restriction and genetic map of the 301 bp fragment from the *E. coli lac* genetic control region. 'i' is the repressor gene, CAP refers to the catabolite gene activating protein binding site (sometimes designated CRP), 'P' denotes the promoter sequence, 'O' is the operator sequence with the repressor binding site, and 'z' refers to the β -galactosidase gene. The sequence is numbered so that the first nucleotide of the mRNA is +1. The lower panel indicates the fragments derived from digestion of the 301 bp fragment with the restriction endonucleases which are designated. The numbers of bp are not additive in some cases because of the presence of "sticky ends". The percentage values in brackets refer to the GC content of the respective fragments.³³ The base compositions were calculated from the known sequence.¹¹ The Alu I and Hae III restriction maps are shown to locate the 95 and 203 bp fragments. However, the 95, 203, and 301 bp fragments were isolated from different recombinant plasmids which were constructed,¹¹ specifically in order to facilitate the preparation of these fragments. For this reason all three fragments contain Eco RI "sticky ends".

a variety of different GC contents ranging from 44% for the 80 bp fragment to 63% for the 101 bp fragment. The 101 bp fragment does not contain any known protein binding site, the 80 bp fragment contains the *lac* repressor binding site, the 95 bp sequence contains the *lac* repressor and the *E. coli* RNA polymerase binding sites and the 188, 203 and 301 bp fragments contain the CAP binding site in addition to the other two sites. The 219 bp sequence contains the CAP binding site. Fig. 1 also shows that the 188 bp fragment is completely embodied in the 203 bp fragment and may therefore serve as a control of the results. The measurements for these two fragments gave indeed the same results.

Fig. 2 shows a 5% acrylamide gel electrophoretic analysis of some of the fragments used in this study. The samples are homogeneous in size and sequence and are free (<2%) of contaminating nucleic acids.

b) CD spectra of the 95 and 101 bp fragments

Fig. 3A displays the CD spectra of the 95 bp fragment in the B-form in standard buffer, shifted towards the C-form in 50% ethanol, and under A-form conditions in 80% ethanol. Whereas the spectrum in 50% ethanol was recorded immediately after mixing the proper amount of ethanol into the DNA stock solution, the A-form spectrum was recorded after equilibrating the mixture containing 80% ethanol at 4°C overnight. Fig. 3B shows the spectra of the 101 bp fragment under the same conditions.

The spectra for these two fragments were chosen for presentation since they have the second lowest (95 bp, 47%) and

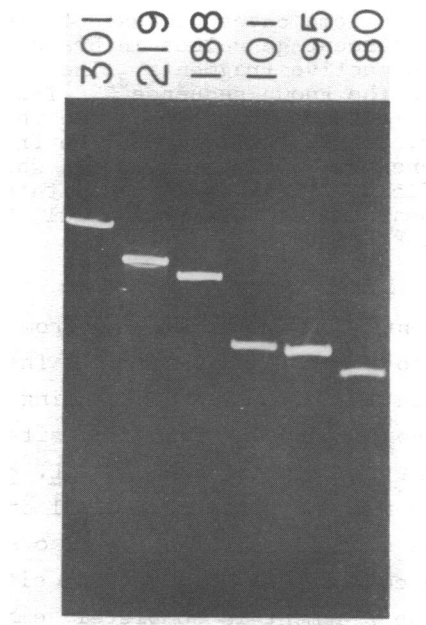


Figure 2. 5% polyacrylamide gel electrophoresis of the fragments used in this study. The numbers refer to the size of the fragments (in bp). The amount of DNA loaded on the gel varied between 0.3 and 0.8 μ g. Characterization and purity of the 203 bp fragment was reported elsewhere⁸.

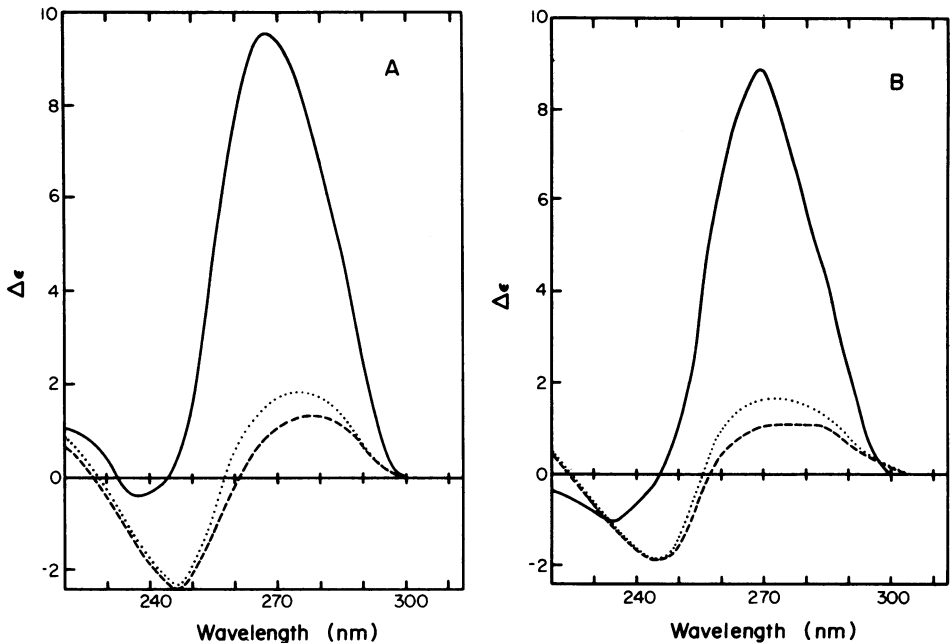


Figure 3. CD spectra of the 95 bp (panel A) and 101 bp (panel B) fragments in standard buffer (dotted line); 1/2 standard buffer - 50% ethanol (v/v) (dashed line); and 1/5 standard buffer - 80% ethanol (v/v) (solid line). The 80% ethanol spectra were taken after equilibrating the samples overnight at 4°C.

the highest (101 bp, 63%) GC content. As mentioned above, the 95 bp fragment contains the *E. coli* RNA polymerase and *lac* repressor binding sites, whereas the 101 bp sequence lacks protein binding sites. Thus, any sequence-dependent difference in the CD properties of the fragments should be revealed by these two species. Complete CD analyses were performed on all other fragments but the primary data is not shown because they lead to the same conclusions.

Prior studies^{22,34} with DNAs from *M. luteus* (72% GC), *E. coli* (50% GC), and *C. perfringens* (31% GC) showed that the long wavelength CD maxima for the B- and C- form is broadened towards the 260 nm side with increasing GC content. This behavior is also found for the fragments (Figs. 3A and B and data

not shown).

At ethanol concentrations below the B→A transition, the positive CD band between 260 and 295 nm is diminished as the ethanol concentration is increased. This is interpreted as a shift of the helical geometry of the DNA toward the C- form, similar to the effect of increasing the salt or the methanol concentration^{25,27} for chromosomal DNAs. However, this interpretation has recently been questioned by new results from fiber x-ray diffraction studies²⁰. Therefore, the cause of this spectral change is unclear.

In 80% ethanol, the general characteristics of the spectra for the two fragments (as well as the other five) resemble those found for chromosomal DNAs under similar conditions^{21,22}. The shapes as well as the amplitudes agree almost exactly.

In order to make obvious any systematic differences between these fragments, the difference spectra ($\Delta\epsilon_{101}$ minus $\Delta\epsilon_{95}$) for all three conditions are shown in Fig. 4. For the

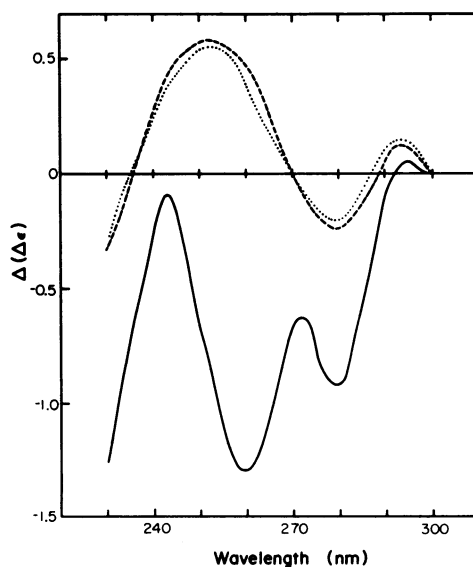


Figure 4. Difference spectra $\Delta\epsilon_{101bp} - \Delta\epsilon_{95bp}$ for standard buffer (dotted line); 1/2 standard buffer - 50% ethanol (v/v) (dashed line); and 1/5 standard buffer - 80% ethanol (v/v) (solid line).

standard buffer and the 50% ethanol conditions, the difference spectra are of the same shape and almost exactly the same amplitude. This indicates that the changes in both DNAs leading to the perturbation of the CD spectra in 50% ethanol are the same. They do not seem to depend on the base sequence or the GC content of the fragments. It is also noteworthy that these difference spectra very closely resemble those measured for chromosomal DNA²², in agreement with the conclusion of the lack of importance of the base sequence for this transition.

In contrast, the difference spectrum in 80% ethanol is of a different shape and polarity than the spectra in 50% ethanol and in standard buffer. This spectrum also differs in shape from the corresponding data for chromosomal DNA²². The interpretation of this observation will be discussed below. Although the shape of the difference spectra for the A-forms in Fig. 4 is reproducible, the $\Delta(\Delta\epsilon)$ values differ by approximately $\pm 30\%$ between repeat experiments.

c) Kinetics of the B \rightarrow A structural transition

The next study was to determine the ethanol concentration required to cause the B \rightarrow A transition for each of the seven fragments. This experiment was performed by mixing the appropriate amount of ethanol with the DNA stock solution and measuring the ellipticity at 270 nm. The titration to higher ethanol concentrations was continued by the stepwise addition of small amounts of ethanol to this solution. This procedure led to large variations of $\Delta\epsilon$ values for ethanol concentrations higher than the B \rightarrow A transition midpoints (see below). Also complete spectra run soon after mixing (within approximately 1 h) were only very poorly reproducible.

It should be noted that this procedure differs from the published method²¹ in which Ivanov *et al.* equilibrated the sheared chromosomal DNA-ethanol mixture overnight before the CD measurement.

Kinetic studies were performed to attempt to resolve these differences. Fig. 5 shows the increase in ellipticity at 270 nm with time after making the 95 bp fragment 80% in ethanol. The CD change was followed approximately 2 min after mixing.

Within the first two minutes the $\Delta\epsilon$ increases about 3.5

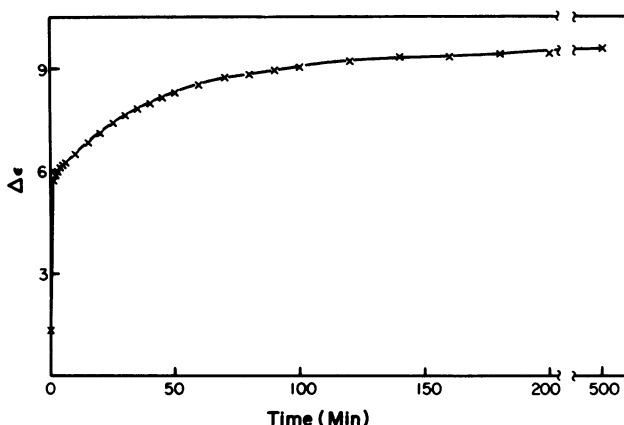


Figure 5. Time dependence of the ellipticity measured for the 95 bp fragment in 1/5 standard buffer - 80% ethanol (v/v). At zero min, the ethanol was mixed into the sample. The $\Delta\epsilon$ for this time was extrapolated from measurements at ethanol concentrations below the B \rightarrow A transition. After 2 min, the CD signal at 270 nm was followed.

fold. The starting value for zero min is extrapolated from the $\Delta\epsilon$ values at ethanol concentrations below the B \rightarrow A transition (\sim 60%). After this fast transition, a second slow increase of the CD signal is observed. The $t_{1/2}$ of this slow transition is 27 min in this case. The identical experiment was repeated with one half the DNA concentration (data not shown); the same shape of the kinetic curve was observed but the $t_{1/2}$ for the slow transition was 62 min. A number of other measurements confirmed the pronounced concentration dependence of the half time of this slow conformational change. The half time of the transition also is rather sensitive to the exact ethanol concentration which detracts from a more quantitative evaluation. Moreover, the data in Fig. 5 infers that no precipitation of the sample had occurred even after 9 h as judged by the stability of the CD signal. If a DNA precipitate had settled to the bottom of the cuvette, this stability would not be found.

All of the other 6 fragments show a kinetic curve like that shown in Fig. 5 for the 95 bp fragment (data not shown).

The comparison of the aggregation of different fragments is hampered by the experimental difficulties stated above for the 95 bp fragment.

Because this strong concentration dependence suggests aggregation of the DNA, sedimentation determinations were performed on the 95 bp fragment under the conditions of the CD measurement. The s value for the 95 bp fragment in 68% ethanol ($s_{\text{obs.}} = 4.75$) was increased by only a factor of 2 over the s value in 50% ethanol ($s_{\text{obs.}} = 2.10$). However, the s value in 80% ethanol ($s_{\text{obs.}} = \sim 160$, varying between 90 and 230 in repeat experiments) was increased approximately 80 fold over the value in 50% ethanol. This observation also strongly suggests that the DNA is aggregated but not precipitated at high ethanol concentrations.

d) Ethanol concentration required to cause the B \rightarrow A transition

All seven fragments were titrated in order to compare the ethanol concentration at the transition midpoints. Because the fast transition (Fig. 5) probably represents the B \rightarrow A conformational change of the DNA double helix, the ellipticity of the samples in the titration experiments was measured immediately after mixing with the desired amount of ethanol. Fig. 6 shows the titration curve for the 95 bp fragment, which is identical in shape to those measured for the other six fragments. Using this method, it is possible to measure reproducibly the end value after the fast transition. It should also be noted that results from a titration done by diluting samples equilibrated at 80% ethanol with standard buffer resulted in the same midpoint concentration.

The ethanol concentration at the midpoint of the transitions for all seven fragments was $72 \pm 2\%$. Since the accuracy for adjusting the ethanol concentration is $\pm 2\%$, the differences between fragments are not significant. The conclusion, therefore, is that the amount of ethanol required to cause the B \rightarrow A transition shows no sequence specificity among these fragments.

DISCUSSION

Most prior CD studies on DNA have been on synthetic poly-

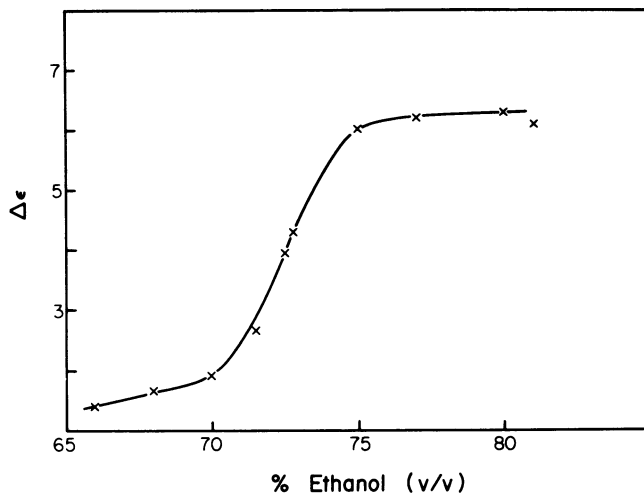


Figure 6. Titration of the 95 bp fragment with ethanol. The CD signal at 270 nm of the sample is plotted versus the ethanol concentration. The ellipticity was read immediately after mixing the ethanol into the sample to minimize contributions from the slow transition (see text).

nucleotides³⁴⁻³⁶, samples of sheared chromosomal DNAs from various sources covering a broad range of GC contents²², or on homogeneous samples of viral DNA which are several thousand bp long^{27,28}. Studies on various synthetic polynucleotides suggested that, at least in some cases, the primary sequence influenced the conformation of the DNA^{5,34,35}. Studies with sheared chromosomal DNAs do not provide information on this notion because they average over all the different sequences present on the genomes. This limitation is overcome in this study by the use of small, sequenced restriction fragments. It is also of advantage to work with physically homogeneous samples. The DNA fragments used in this study cover a difference in GC content of approximately 20%. Furthermore, the distribution of nucleotides within the fragments is rather inhomogeneous³³.

The CD spectra of the B-form at low ionic strength, the more C-like form in 50% ethanol and the A-form in 80% ethanol for the seven fragments agree well with the literature data for

DNA with similar GC content^{22,27}. The difference spectra in low salt and 50% ethanol are qualitatively the same as those measured for *M. luteus* and *C. perfringens* DNA²². The only minor difference between these data is that the positive difference around 260 nm shows a split band for the random DNA whereas no significant splitting could be detected for the fragments.

In contrast, the A-form difference spectrum is not of the same shape as the literature data²². The conclusion for the random DNA was that, regardless of the GC content, both DNA samples undergo the same changes reflected by the same shape of the difference spectra in all forms. This clearly cannot be concluded for the fragments studied here. Instead, a different state of the A-form for both fragments seems to be present in this case. This observation will be discussed below in more detail. The only difference in experimental procedures is that we used ethanol instead of $\text{CF}_3\text{CH}_2\text{OH}$ ²² to prepare the A-form samples.

Due to the homogeneity and small size of these samples, one expects a decreased tendency for aggregation as compared to sheared samples^{27,37,38}. Therefore, the ethanol in this study was added at once to adjust the final concentration. The spectra at 80% ethanol were then taken after equilibrating the mixture for approximately 15 h. From the general agreement of the A-form spectra reported here with the literature data^{21,22} it is concluded that the different DNAs exist in similar conformations at 80% ethanol, and that the aggregates do not differ greatly in molecular weight. This conclusion is further confirmed by the fact that the A-form spectra remained unchanged when the equilibration of the sample was done by adding ethanol slowly and stepwise.

A drastic reduction of the ellipticity of PM2 DNA at 270 nm was reported recently when the ethanol concentration was increased to 90%²⁷. At concentrations of up to 82% ethanol, we do not find any drop in the CD signal over a 15 h time period.

Kinetic studies were performed after mixing the total amount of ethanol at once into the sample. Upon adjusting the ethanol concentration to 80%, the DNA undergoes at least one

fast reaction in the first two minutes, followed by a slow step. The fast step is probably the monomolecular B→A transition. The slow reaction may reflect an aggregation of the DNA helices. This interpretation is supported by the strong concentration dependence of the slow reaction. It is, of course, impossible to conclude from these data that the fast reaction is concentration independent. Nevertheless, we assume that the DNA adopts the A-form prior to aggregation. The sedimentation data confirm the conclusion that the DNA is aggregated after the slow reaction has taken place. Although the *s*-values at low ionic strength do not depend in a straight-forward way on the molecular weight, the 80-fold increase clearly indicates aggregation. On the other hand, the DNA aggregates do not settle even after 15 h and the ellipticity of the sample remains unchanged. We therefore conclude that the DNA is aggregated, rather than precipitated. The A-form difference spectrum in Fig. 4 contains the contributions arising from aggregation, which may explain its deviation in shape from the B- and C-form difference spectra. The variation of the *S*_{obs.} values at 80% ethanol, the dependence of *t*_{1/2} for the slow transition on the ethanol concentration in addition to the DNA concentration, and the reduced reproducibility of the A-form difference spectra suggest that the formation of DNA aggregates is not very specific and might be sensitive to slight differences in the ethanol as well as DNA concentrations.

A recent independent study³⁸ indicates that the *s* value of sheared calf thymus and T7 DNAs of different molecular weight increases at ethanol concentrations of 75% or higher. From the independence of the CD signal at 260 nm on the preparation of the sample, these authors conclude that the CD spectrum is insensitive to aggregation and depends only on the B- to A-form transition³⁸. Fig. 5 herein clearly shows that the CD signal at 270 nm changes upon aggregation of these DNA fragments. The lowest molecular weight of the seven fragments is 54,800 d (80 bp with a total of six bases in "sticky ends"). Thus, it may be necessary to study even smaller fragments to verify the molecular weight dependence of the aggregation suggested in ref. 38.

The titration of the B→A transition was designed to detect only the fast conformational change. The measurements did not reveal any increased scatter of the data points around the smooth transition curves, which would have indicated varying contributions from aggregation. It was, therefore, not necessary to do this measurement by decreasing the ethanol concentration in the preequilibrated A-form sample, as performed previously²¹. In a control experiment, the transition midpoint of the B→A transition was the same. It is also identical to the one found for chromosomal DNA regardless of its GC content^{21,17}. Hence, we conclude that the B→A transition is sequence independent.

In summary, upon adding ethanol to 80%, the DNA undergoes a conformational transition to the A-form in a fast, probably monomolecular, reaction. This is followed by a slow aggregation, leaving the DNA in an aggregated, but probably not precipitated, form. The lack of influence of the different sequences in this study on the dehydrating conditions suggests that the driving force for these structural transitions is in the polynucleotide backbone rather than in the primary sequence¹⁷.

It has been suggested that during transcription the DNA adopts the A-form in the complex with RNA-polymerase³⁹. From the results of this study it seems that the lac-promoter sequence does not have any preference for the A-form.

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